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Transcriptome Analysis Identifies Regulators of Hematopoietic Stem and Progenitor Cells

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SUMMARY

Hematopoietic stem cells (HSCs) maintain blood homeostasis and are the functional units of bone marrow transplantation. To improve the molecular understanding of HSCs and their proximal progenitors, we performed transcriptome analysis within the context of the ImmGen Consortium data set. Gene sets that define steady-state and mobilized HSCs, as well as hematopoietic stem and progenitor cells (HSPCs), were determined. Genes involved in transcriptional regulation, including a group of putative transcriptional repressors, were identified in multipotent progenitors and HSCs. Proximal promoter analyses combined with ImmGen module analysis identified candidate regulators of HSCs. Enforced expression of one predicted regulator, *Hlf*, in diverse HSPC subsets led to extensive self-renewal activity ex vivo. These analyses reveal unique insights into the mechanisms that control the core properties of HSPCs.

INTRODUCTION

Hematopoietic stem cells (HSCs) reside at the apex of the hematopoietic hierarchy and generate the entire repertoire of highly specialized hematopoietic effector cells by differentiating through a succession of increasingly committed progenitors. HSCs are the only hematopoietic cell type that can differentiate into all blood lineages and self-renew for life. These properties, along with HSCs' remarkable ability to engraft conditioned recipients upon intravenous transplantation, have established the clinical paradigm for the application of stem cells in regenerative medicine. Indeed, HSC transplantation is routinely used to treat a variety of hematological conditions, including leukemia, multiple myeloma, severe combined immunodeficiency, and myelodysplastic syndrome. Nonetheless, HSC transplantation remains a relative high-risk procedure, with the most significant factor contributing to the success of the procedure being the size of the transplanted graft (Siena et al., 2000). Enormous efforts have therefore been mounted to develop methods for expanding HSCs ex vivo, although these efforts have not yet translated to the clinic. A greater understanding of the molecular mechanisms underlying HSC fate and function will undoubtedly inform strategies for the therapeutic manipulation of these cells, and may also improve our understanding of hematopoietic malignancies derived from stem cells.

The ability to purify HSCs to near homogeneity opens the door for their precise molecular characterization by microarray analysis. This approach is particularly useful for studying HSCs because it allows for the simultaneous, quantitative detection of entire transcriptomes from these rare cells. Although prior microarray studies have provided useful insights into HSC biology (Chambers et al., 2007; Forsberg et al., 2005, 2010; Rossi et al., 2005), it has proved challenging to cross-analyze data due to differences in experimental designs and technical methodologies. The ImmGen Project overcomes many of these limitations by generating transcriptome data from stem cells, defined progenitors, and various effector cells, using unified protocols of cell sorting, RNA extraction, unamplified sample preparation, and a common facility for microarray processing (Heng and Painter, 2008; Painter et al., 2011). Additional advantages of the ImmGen approach include a wider breadth of assayed hematopoietic cell types and states (~250), increased statistical power through array number

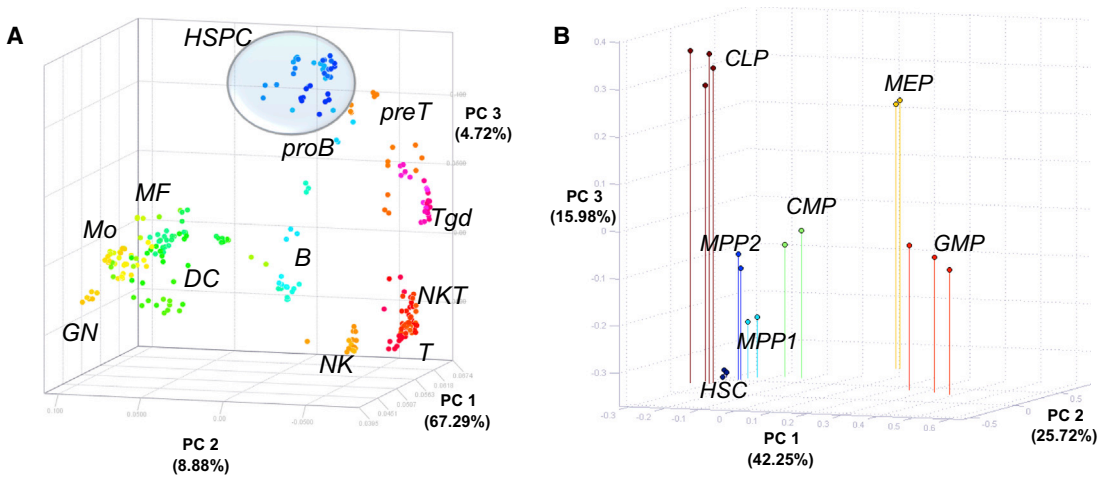

Figure 1. Population Distances Define HSPCs in Transcriptional Space

(A) Population-distance analysis of microarray data presented in three principal components (PCs 1–3). Each point represents a single array. Cell types are color-coded. B, B cells; DC, dendritic cells; GN, granulocytes; MF, macrophages; Mo, monocytes; NK, NK cells; NKT, NKT cells; preT, T cell precursors; proB, B cell precursors; T, T cells; Tgd, γδ T cells.

(B) Population-distance analysis of HSPC subsets including HSCs, MPPs (MPP1 and MPP2), and oligopotent progenitors (CLP, CMP, MEP, and GMP).

See also Figure S1.

(~700 total), and utilization of the Affymetrix GeneChip Mouse Gene ST 1.0 microarray platform, which includes probes for >24,500 coding and >1,300 noncoding transcripts.

Here, we used the breadth of the ImmGen data set to delineate genes and regulators of the primitive hematopoietic cells, bringing to light conceptual advances at three levels of resolution: (1) hematopoietic stem and progenitor cells (HSPCs), (2) multipotent stem and progenitor cells, and (3) HSCs. All HSPCs showed enriched expression of metabolic growth- and proliferation-associated genes, which paradoxically were also expressed in quiescent HSCs. Genes encoding transcription factors, including a group of Kruppel-associated box (KRAB) domain-containing CH3 zinc-finger proteins that are predicted to function as transcriptional repressors, were enriched in multipotent progenitors (MPPs) and HSCs. Exposure to clinically relevant mobilizing stimuli led to alterations in the expression of HSPC regulators, as well as membrane and extracellular matrix proteins and proteases. Proximal promoter analysis of genes identified in steady-state HSPCs and mobilized HSPCs (moHSPCs) revealed enrichment of motifs representing putative binding sites for both known and unknown stem cell regulators, and ImmGen module analysis of HSC-enriched genes independently identified potential regulators. Enforced expression of one putative regulator, *Hlf*, resulted in robust induction of a primitive immunophenotype, sustained colony-formation activity, and enhanced self-renewal in a number of progenitor subsets ex vivo.

RESULTS

Comparative Transcriptional Distances between Primitive HSPCs

The generation of effector blood cells from HSCs proceeds through a series of downstream progenitors with increasingly restricted potential (Bryder et al., 2006). The most proximal progenitors to HSCs are MPPs, which retain full lineage potential but lack long-term self-renewal potential. As MPPs differentiate, they give rise to oligopotent progenitors of either lymphoid or myeloid effector cells. To generate transcriptome data from primitive subsets, we sorted HSCs, MPPs (MPP1 and MPP2), and oligopotent progenitors (common myeloid progenitor [CMP], granulocyte-macrophage progenitor [GMP], megakaryocyte-erythroid progenitor [MEP], and common lymphoid progenitor [CLP]) to a high degree of purity (for sorting details, see Table S1 available online and http://www.immgen.org/index_content.html) and subjected them to ImmGen expression profiling and quality-control pipelines (Heng and Painter, 2008). Hereafter, we refer collectively to these primitive subsets as HSPCs. Principal component analysis (PCA) was performed on the 20% most variable genes between HSPCs and their downstream progeny (Figure 1A). Strikingly, all HSPC subsets clustered closely together in relation to their downstream progeny, indicating that hematopoietic progenitors as functionally diverse as HSCs, CLPs, and CMPs share gene expression properties that commonly define them in transcriptional space.

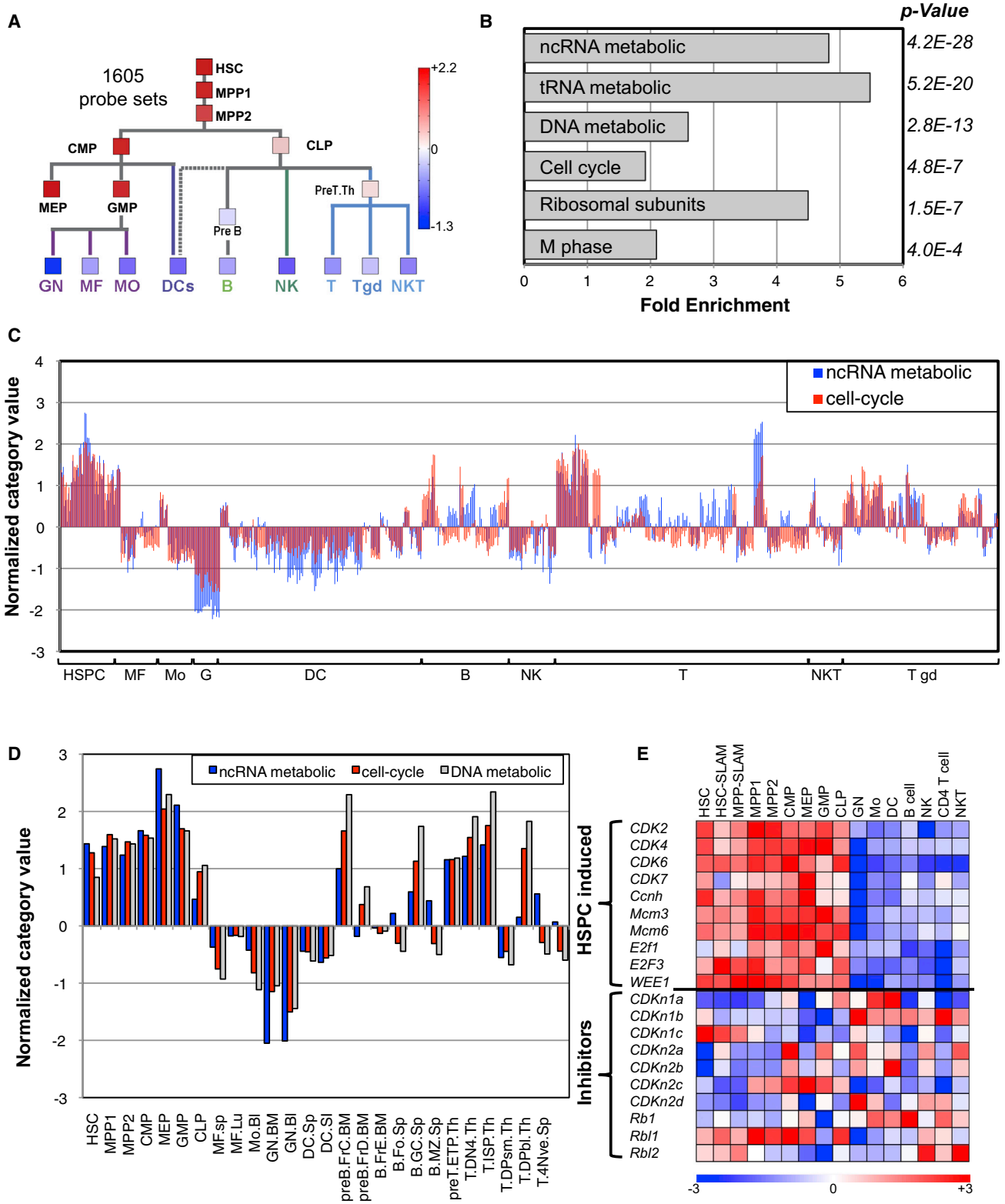

Figure 2. HSPCs Are Enriched for Gene Sets that Enable Transit Amplification

(A) Reduced representation of hematopoiesis showing normalized and averaged values of 1,605 HSPC-enriched genes.

(B) DAVID analysis showing enriched categories, with adjusted p value (Benjamini).

(legend continued on next page)

We next interrogated the transcriptional relationships among hematopoietic progenitors by performing PCA analysis using the 20% most variable genes between the HSPC subsets to define transcriptional distances. In agreement with established functional relationships (Bryder et al., 2006), MPP1 were positioned most proximal to HSCs, followed by MPP2, whereas oligopotent lymphoid and myeloid progenitors radiated farther along the principal components (Figure 1B).

HSPCs Are Transcriptionally Enriched for Genes Associated with Transit Amplification

Though HSPCs represent a group of progenitors with divergent functional attributes, the relatedness of their transcriptomes (Figure 1A) prompted us to determine whether we could identify a set of genes commonly expressed across diverse HSPC subsets. We therefore analyzed the combined HSPC subsets in comparison with their downstream hematopoietic progeny by one-way ANOVA (false discovery rate [FDR] < 5%, $p < 1 \times 10^{-5}$) and identified 1,605 genes with enriched expression in HSPCs (Figure S1A; Table S2). A reduced representation of relative expression, averaged from all 1,605 genes, showed high expression in HSCs, MPPs, and oligopotent myeloid progenitors, and a lower level of induction within CLPs (Figure 2A). We next tested for functional enrichment in the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (http://david.abcc.ncifcrf.gov/), which revealed significant enrichment for genes associated with metabolic growth (ncRNA-metabolic, tRNA-metabolic, and Ribosomal subunits) and proliferation (cell cycle, DNA-metabolic, and M-phase; Fisher's exact test, FDR < 4×10^{-4}; Figure 2B), consistent with the high cycling activity and transit amplification potential of these progenitors.

To visualize the relative expression of genes identified by ANOVA across the ImmGen data set, we normalized the expression values of the genes in either the ncRNA metabolic or cell cycle groups and plotted the average expression for each cell type (Figure 2C). This analysis showed that in addition to HSPCs, these gene sets are also highly expressed in early B and T cell progenitors (Figure 2C; Figures S1B and S1C), in line with the proliferative potential of these precursors (Carpenter and Bosselut, 2010). In contrast, effector cells such as granulocytes, dendritic cells, and natural killer (NK) cells showed markedly lower expression, consistent with their terminally differentiated state.

Interestingly, HSCs showed relatively high expression of metabolic growth and proliferation gene sets (Figure 2C), despite the fact that they are largely quiescent in adults (Bowie et al., 2006; Rossi et al., 2007; Wilson et al., 2008). To further explore this apparent paradox, we plotted the relative expression of genes in the ncRNA-metabolic, cell cycle, and DNA-metabolic categories in a limited subset of cell types, reasoning that this might allow us to discriminate between genes that encompass both positive and negative regulators of cell proliferation (found in the cell cycle gene sets) and genes that are more tightly linked to DNA synthesis (found in the DNA metabolic gene sets; Figure 2D). Surprisingly, although the HSCs showed a slight relative decrease in expression of genes associated with DNA metabolism in comparison with other HSPC subsets, they nonetheless exhibited relatively high expression of genes in these categories. These data raise the possibility that even though they reside predominantly in the quiescent G0 phase of the cell cycle, HSCs are nonetheless transcriptionally poised to enter the cell cycle by expression of genes that mediate cell-cycle progression. This postulate implies that active maintenance of quiescence is a requisite feature of adult HSCs, a notion that has been borne out in studies that have defined regulators that hold HSCs in a quiescent state. To explore this concept further, we examined the expression of a subset of positive and negative regulators of the cell cycle (Figure 2E). Interestingly, whereas HSCs clearly showed robust expression levels of cell-cycle drivers such as *Cdk2*, *Cdk4*, and *Cdk6*, the only canonical Cdk inhibitor with high expression in HSCs was *Cdkn1c*, which encodes p57, a protein that was recently shown to regulate HSC quiescence (Matsumoto et al., 2011; Zou et al., 2011). The Rb family members also showed expression (albeit nonpreferential) in HSCs (Figure 2E), in agreement with their combined role in regulating HSC quiescence (Viatour et al., 2008).

Cumulatively, these results demonstrate that HSPCs exhibit elevated expression of genes consistent with their high cycling activity, and suggest that HSC quiescence is a poised state in which genes and pathways required for cell-cycle entry and growth are expressed.

Identification of a Group of CH3 Zinc-Finger KRAB Domain-Containing Transcriptional Repressors in Multipotent Stem and Progenitor Cells

To identify genes and pathways enriched in hematopoietic multipotency, we analyzed multipotent stem/progenitors

(C) Normalized and averaged values for the indicated categories across the ImmGen data set. Cell types were grouped as indicated.
(D) Normalized and averaged values for the indicated categories in ImmGen data sets.
(E) Heatmap of positive and negative regulators of cell cycle in the indicated cell types.
See also Figure S2.

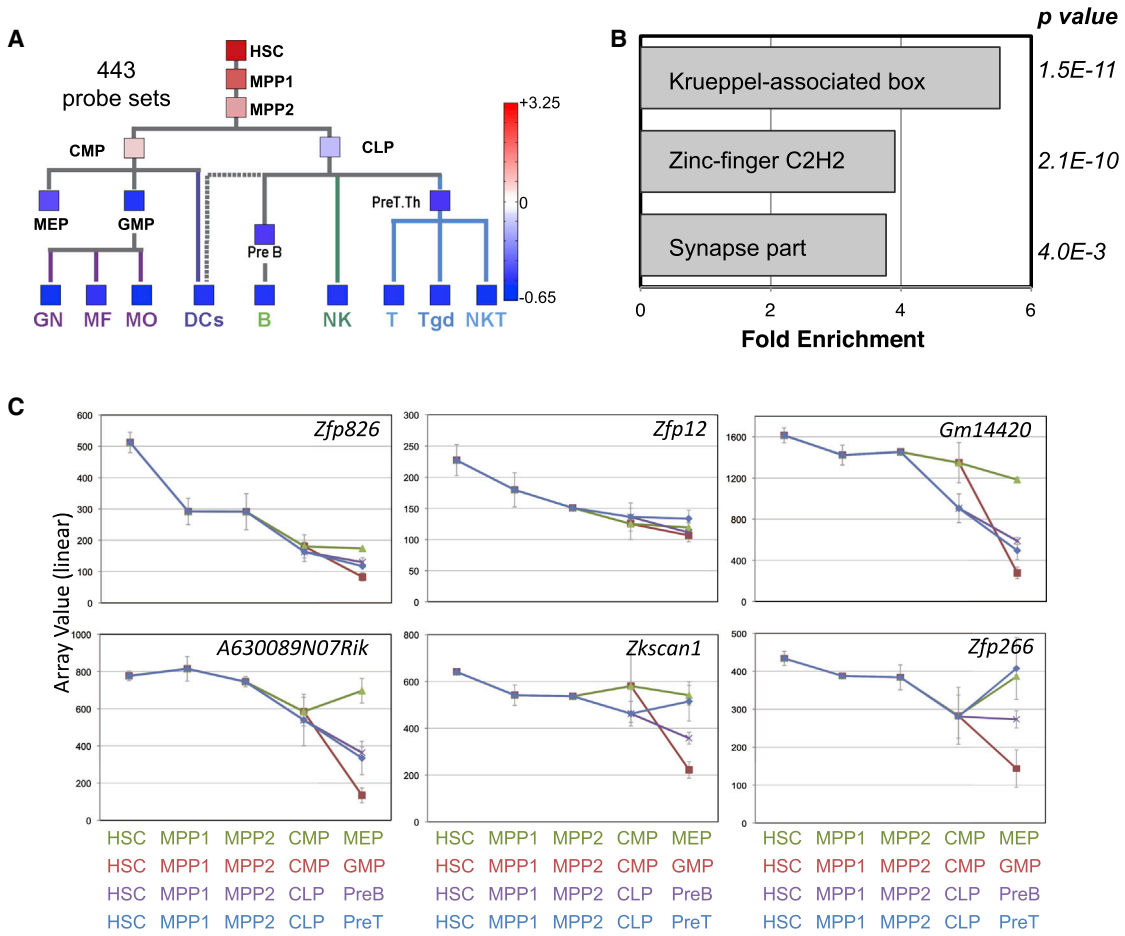

Figure 3. Hematopoietic Multipotent Stem and Progenitor Cells Express a Family of KRAB Domain-Containing Zinc-Finger Transcriptional Repressors

(A) Reduced representation of hematopoiesis showing normalized and averaged values of 433 MPP-enriched genes.

(B) DAVID analysis showing enriched categories, with adjusted p value (Benjamini).

(C) Graphs showing the linear values (averaged array replicates ± SEM) of the indicated genes along differentiation trajectories from HSC to MEP (green), GMP (red), PreB (purple), and PreT (blue). Biological replicates: n = 2 (MPP1, MPP2, and MEP), n = 3 (HSC, CMP, GMP, PreB, and PreT), and n = 4 (CLP).

See also Figure S3.

(HSCs, MPP1, and MPP2) as a group in comparison with their downstream progeny, and identified 443 genes with enriched expression (one-way ANOVA, FDR < 5%, $p < 1 \times 10^{-4}$; Table S3; Figure S2). Reduced representation of expression showed the highest relative expression in HSCs, followed by MPP1s and MPP2s (Figure 3A). DAVID analysis revealed a significant overrepresentation of genes encoding KRAB domain-containing proteins and C2H2 zinc-finger domain-containing proteins (Figure 3B). When present in proteins that also contain DNA-binding domains, KRAB domains canonically function to recruit transcriptional repressors (Urrutia, 2003), and since all of the KRAB domain-containing proteins we identified also contain C2H2 zinc-finger DNA-binding domains, it is predicted that these proteins function as transcriptional repressors. To visualize their expression at increased resolution, we focused our analysis on how their expression levels change during differentiation between stem cell and defined downstream progenitor cell populations (Figure 3C; Figure S3). The preferential expression of these putative transcriptional repressors in primitive progenitors that possess multilineage differentiation capacity raises the possibility that they may be involved in maintaining hematopoietic multipotency through KRAB-mediated suppression of lineage commitment pathways in a general (e.g., *Zfp826* and *Zfp12*) or lineage-specific (e.g., *Gm14420*, *A630089N07Rik*, *Zkscan1*, and *Zfp266*) manner.

Transcriptional Regulation of HSCs

We next sought to identify genes and pathways that might uniquely regulate HSCs within the hematopoietic system. To achieve this, we compared the transcriptome of HSCs with all other hematopoietic cell types in the ImmGen data set and identified 322 genes with enriched expression in HSCs (one-way ANOVA, FDR < 5%; Figures 4A and 4B; Table S4). Functional annotation by DAVID showed that most genes could be grouped into a limited number of categories, whereas 43% (138/322) of the identified HSC genes remained uncharacterized in any cell type (Figure 4B). The 322 HSC-enriched genes were significantly enriched for KRAB domain-containing proteins and C2H2 zinc-finger transcription factors, as observed in the broader multipotent stem and progenitor cell analysis. In total, 51 of the 322 HSC-enriched genes were identified as transcription regulators (Figure 4C) whose HSC-enriched expression proved to be conserved between mouse and human (Figure 4D), and included known HSC regulators such as *Meis1*, *Mecom/Evi-1*, *Ndn*, *MycN*, and *HoxA9* (Figures 4C and 4D). To explore the interrelationships of these factors, we constructed a functional gene network using a context likelihood of relatedness (CLR)-based method (Faith et al., 2007) and the entire ImmGen data set to derive connections between genes in this network representing nonrandom and statistically significant dependencies. Strikingly, of the 51 HSC-enriched transcription factors we identified, 48 segregated into two distinct clusters (Figure 4E). Interestingly, all factors that were previously reported to operate functionally in HSCs fell into one network cluster, suggesting that these genes may be under a common regulatory architecture (Figure 4E).

To clarify regulators of HSC-specific gene expression, we next used de novo motif discovery (MEME) (Machanick and Bailey, 2011) to analyze the proximal promoters of the 322 HSC-enriched genes, defined as ±1,000 bp from the transcription start sites (TSSs). We identified four motifs, which TOMTOM analysis recognized as putative binding sites of a number of transcription factors (Figure 4F). The most significant motif is a putative binding site of EGR1, which was previously demonstrated to regulate HSC quiescence and retention in bone marrow (BM) (Min et al., 2008). The second motif is a predicted binding site for SOX4, which is reported to enhance murine HSC reconstitution potential (Deneault et al., 2009). The third motif is a predicted binding site for aryl hydrocarbon receptor (AHR), which is striking in light of a recent report demonstrating ex vivo expansion of HSCs using a purine derivative that acts as an AHR agonist (Boitano et al., 2010). The fourth motif is predicted to bind STAT1, which is required for interferon-induced activation of HSCs (Essers et al., 2009).

To further explore the potential regulatory network of HSCs, we utilized module analysis (http://www.immgen.org/ModsRegs/modules.html), which identifies putative transcriptional regulators based on coexpression across the ImmGen data sets. This analysis was undertaken with the broader ImmGen data set that also includes nonhematopoietic cell types (e.g., stromal and endothelial cells). Four modules were significantly enriched for the HSC-induced genes (hypergeometric, $p < 0.001$; Figure 5A), and each showed a pattern of high expression in stem cells and downregulation upon hematopoietic differentiation. Interestingly, the most enriched module (#40) also showed relatively high expression of a subset of HSC genes in endothelial cells (Figure 5B; Figure S4A). This unexpected finding may reflect the developmental origin of HSCs, which are derived from a population of fetal hemogenic endothelial cells (Dzierzak and Speck, 2008). The module analysis also predicted 32 regulators for the four HSC-enriched modules (Figure 5C; Figure S4B) and included STAT1 and SOX4, which we had identified based on enriched sequence motifs (Figure 4F). Some of the predicted regulators (e.g., *HoxA9* and *Mecom*) showed restricted expression to the primitive hematopoietic compartment, whereas others showed broader expression. The latter group included established HSC regulators, such as *Gata2*, *MycN*, and *Erg*, that showed high expression not only in HSCs but also in endothelial cells (Figure 5C), consistent with their established functional roles in both cell types (Göttgens et al., 2002; Linnemann et al., 2011; Ng et al., 2011; Sato, 2001). Interestingly, the four enriched transcription factor binding motifs we identified in HSCs (Figure 4F) are predicted to bind factors that are expressed in both HSCs and endothelial cells (*Egr1*, *Sox4*, *Ahr*, and *Stat1*), suggesting a shared regulatory program.

G-CSF Mobilization Induces Common Transcriptional Changes in HSCs and MPPs

In adult mice and humans, a small percentage of HSCs and progenitor cells migrate periodically from the BM niche into the circulation (Massberg et al., 2007; Min et al., 2008; Wright et al., 2001b). The frequency of HSCs in the circulation increases significantly in response to inflammation and following administration of mobilizing agents. In particular, treatment of mice or humans with a combination of cyclophosphamide/granulocyte colony-stimulating factor (G-CSF; Cy/G) drives rapid proliferation, expansion, and migration of HSPCs from the BM to peripheral hematopoietic compartments (Morrison et al., 1997; Neben et al., 1993; Passegué et al., 2005), and mobilization is routinely used in clinical practice to collect cells for transplantation. However, the molecular regulators that control HSC expansion and migration during this process remain elusive.

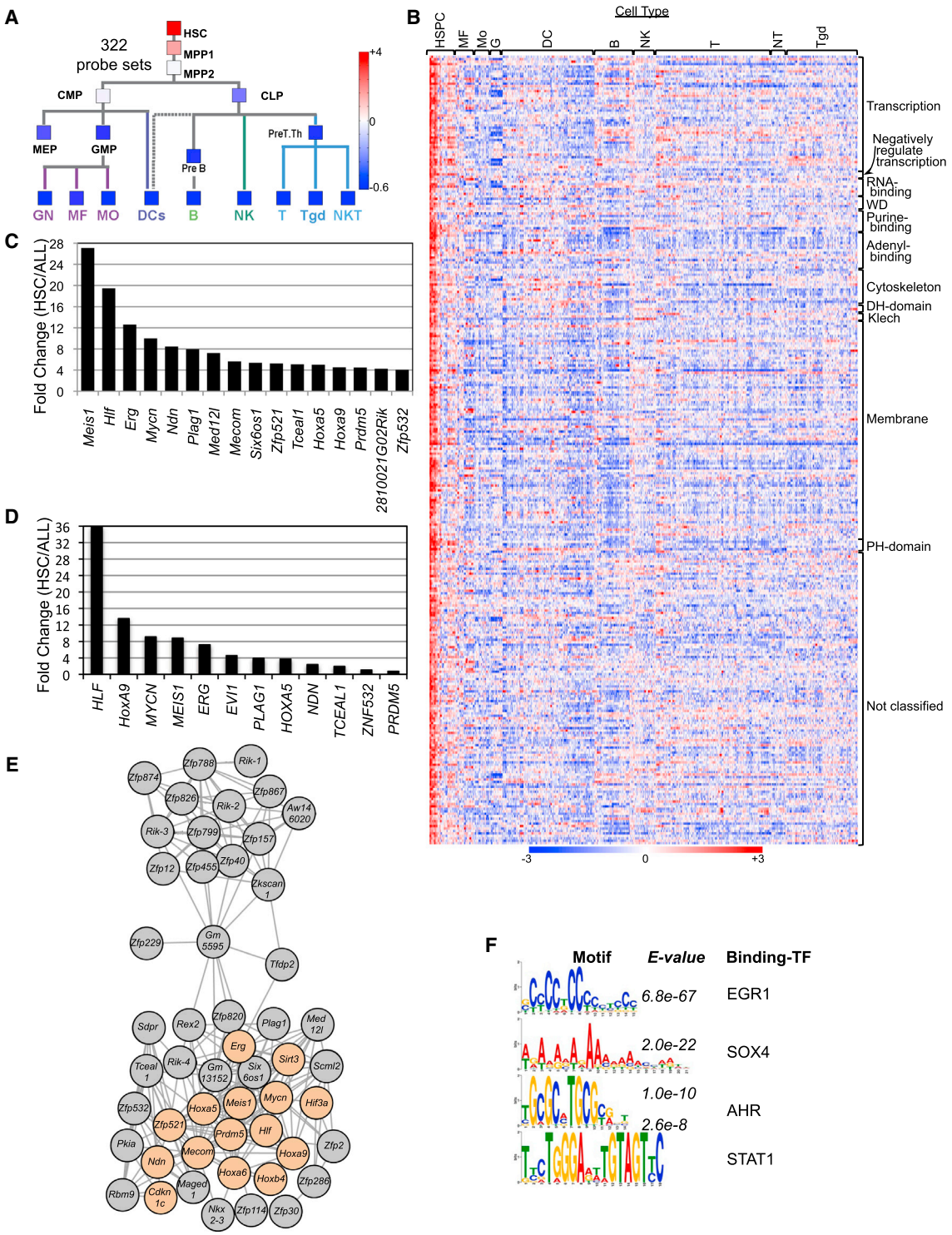

Figure 4. Identification of HSC-Specific Transcriptional Regulators

(A) Reduced representation of hematopoiesis showing normalized and averaged values of 322 HSC-enriched genes.

(B) Heatmap of all HSC-enriched genes across hematopoiesis. Functional classification as determined by DAVID.

(C) Expression of transcriptional regulators enriched (>4-fold) in murine HSCs presented as a ratio of mean expression in HSCs over the mean expression in all other ImmGen cell types.

(D) Expression of the orthologs in (C) in human HSCs (Novershtern et al., 2011).

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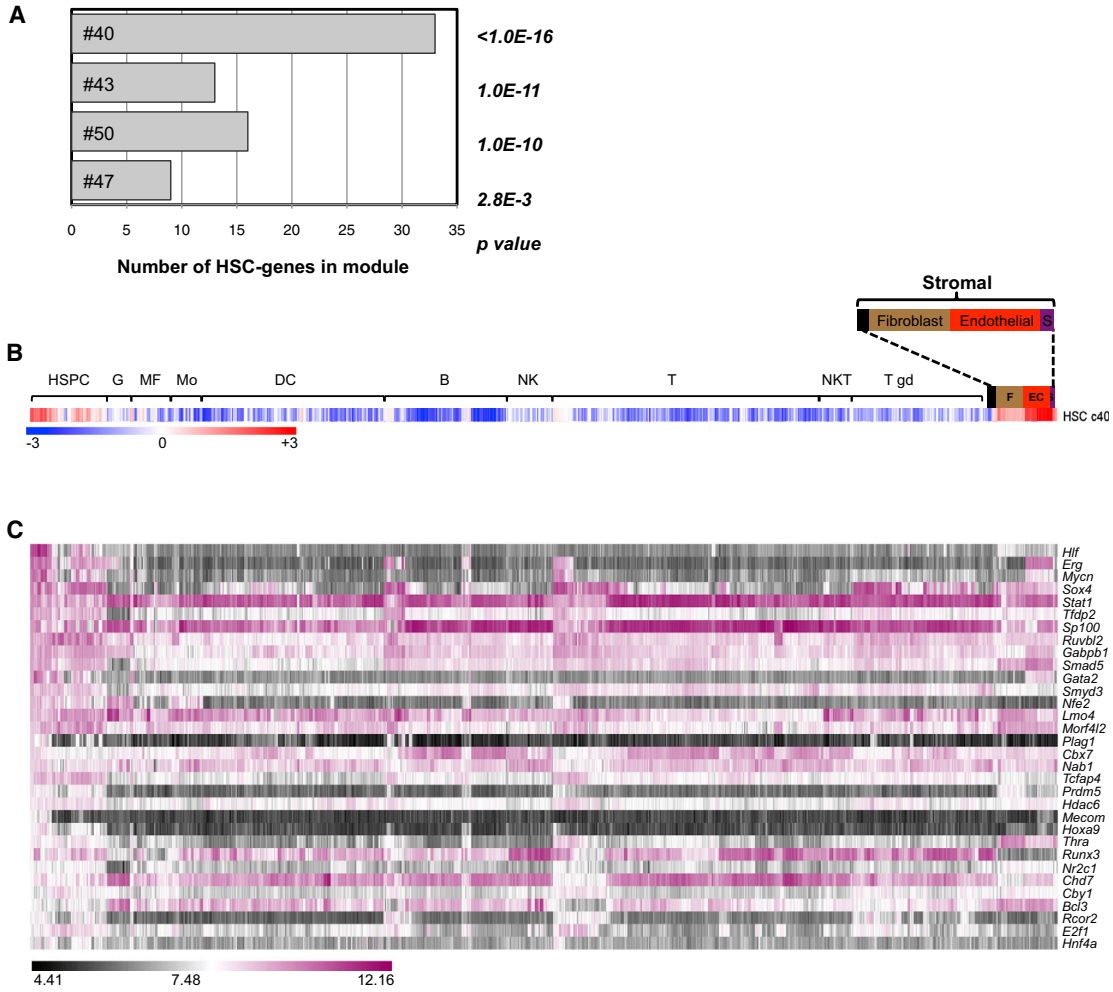


Figure 5. ImmGen Module Analysis Identifies Putative Regulators of HSCs

(A) Graph showing modules (identifier numbers) significantly enriched with HSC-specific genes. Number of common genes and hypergeometric p values are indicated.

(B) Heatmap showing the averaged normalized expression of HSC genes in module #40.

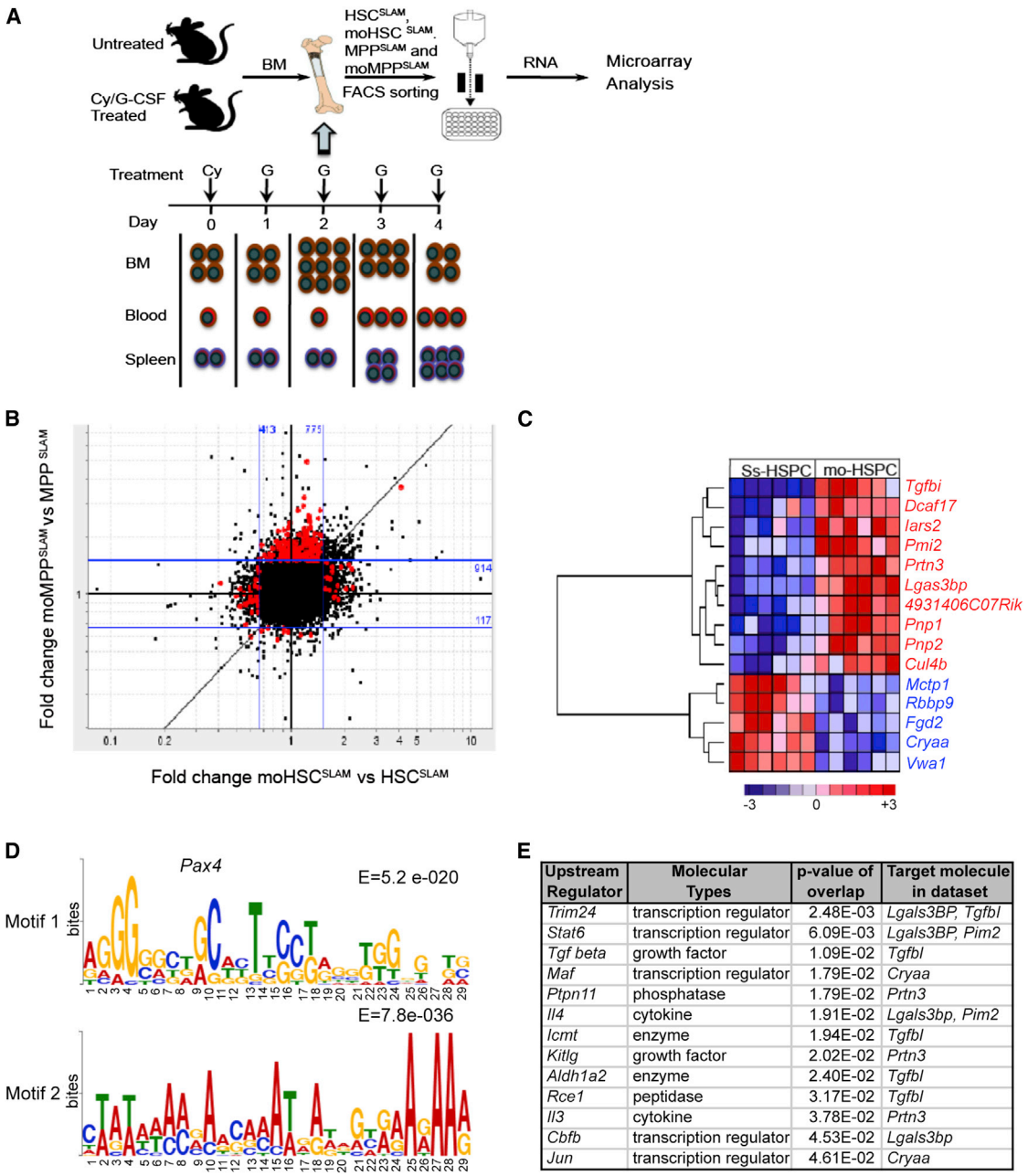
(C) Absolute expression of HSC regulators predicted by ImmGen module analysis. Log_2 values are shown.

See also Figure S4.

HSC^{Slam} and MPP^{Slam} subsets were harvested from Cy/G-treated mice (referred to hereafter as $moHSC^{Slam}$ and $moMPP^{Slam}$, respectively; Figure 6A), and RNA harvested from these cells was compared with RNA extracted from steady-state HSC^{Slam} and MPP^{Slam} (Table S1). Notably, the cell purification strategy used for these mobilization analyses was different from the one used in the previous analyses (Figures 1, 2, 3, 4, and 5), due to the availability of existing functional data that validated these marker sets for isolation of the relevant cell populations from mobilized mice. These samples were also processed with an amplification step and therefore were analyzed separately from the broad ImmGen data set. Importantly, despite the differences in immunophenotype, multiparameter fluorescence-activated cell sorting analyses and mean class expression analyses revealed that SLAM-code (Kiel et al., 2005)

(E) Connectivity map based on correlated expression showing the 51 identified HSC-enriched transcriptional regulators, with known regulators of HSCs highlighted in orange. TF1 = 2810021G02Rik, TF2 = 2610008E11Rik, TF3 = A630033E08Rik, and TF4 = 10305D13Rik.

(F) Significantly enriched sequence motifs ± 1,000 bp of TSS in HSC-enriched genes, showing enrichment values (E values) and predicted binding factors.



E

Upstream Regulator	Molecular Types	p-value of overlap	Target molecule in dataset
Trim24	transcription regulator	2.48E-03	*Lgals3BP, Tgfbl*
Stat6	transcription regulator	6.09E-03	*Lgals3BP, Pim2*
Tgf beta	growth factor	1.09E-02	*Tgfbl*
Maf	transcription regulator	1.79E-02	*Cryaa*
Ptpn11	phosphatase	1.79E-02	*Prtn3*
Il4	cytokine	1.91E-02	*Lgals3bp, Pim2*
Icmt	enzyme	1.94E-02	*Tgfbl*
Kitlg	growth factor	2.02E-02	*Prtn3*
Aldh1a2	enzyme	2.40E-02	*Tgfbl*
Rce1	peptidase	3.17E-02	*Tgfbl*
Il3	cytokine	3.78E-02	*Prtn3*
Cbfb	transcription regulator	4.53E-02	*Lgals3bp*
Jun	transcription regulator	4.61E-02	*Cryaa*

Figure 6. MoHSPCs Express a Defined Gene Signature

(A) Schematic of the Cy/G treatment used to mobilize HSPCs. Mice were injected with a single dose of cyclophosphamide (Cy; 4 mg/mouse, i.p.), followed by two daily G-CSF (G; 5 μg/mouse) injections (D2 Cy/G treatment). HSC^{Slam} and MPP^{Slam} were sorted from untreated and D2 Cy/G-treated mice for RNA extraction and microarray hybridization.

(B) Multiplot analysis to identify differentially expressed genes between each comparison (Hochberg test; FDR < 10%, fold change > 1.5).

(C) Heatmap of differentially expressed genes in moHSPC versus steady-state HSPC (FDR < 10%).

(D) Statistically significant transcription factor binding motifs (TFBs; in the upstream regulatory region and TSS [±1,000 bp]) of differentially expressed genes. The putative TF family binding motif and the p value before the null model correction are noted.

(E) Table of the known upstream regulators of genes in the data set identified by the Ingenuity knowledge base ($p < 0.05$, right-tailed Fisher's exact test).

See also Figures S5 and S6.

HSCs (LSKCD48-CD150$^+$) showed significant overlap with HSCs defined as LSKFlk2-CD34$^-$ (Figure S5A), and expression profiling revealed that the vast majority of genes are similarly expressed in HSCs purified by either strategy (Pearson correlation = 0.997; Figure S5B), with only 24 probe sets exhibiting significantly differential expression (FDR < 10%, fold change > 2; Figure S5C). Moreover, PCA of the 20% most variable genes across these populations showed that HSC^{Slam} and MPP^{Slam} positioned closely to the LKSCD34-Flk2$^-$ HSCs and MPP1s, respectively (Figure S5D), consistent with the previously ascribed immunophenotypic and functional overlap of these populations (Bryder et al., 2006).

Analysis of moHSPCs was performed at day 2 of the mobilization protocol, the peak of Cy/G-induced HSC expansion (Wright et al., 2001a, 2001b), when animals typically show a 3- to 5-fold increase in HSPC number (Forsberg et al., 2010; Passegué et al., 2005). We first considered in aggregate the expression patterns of steady-state HSPCs and moHSPCs. This analysis revealed 15 genes exhibiting differential expression (FDR < 10%) (Figure 6B and 6C; Table S5), and of note was the upregulation in extracellular and transmembrane proteases, including *Prtn3*, which encodes a leukocyte serine protease (Proteinase 3) that degrades elastin, fibronectin, laminin, vitronectin, and collagen IV, and has been suggested to act as a "path clearer" for neutrophil migration (Kuckleburg et al., 2012). Although previous studies have implicated activated immune cells as the primary effectors of proteolysis during HSPC mobilization, the upregulation of matrix proteases in moHSPCs suggests that autocrine proteolysis may also be important. To identify candidate transcriptional regulators of the moHSPC genes, we performed MEME analysis of proximal promoters (±1,000 bp of the TSS) of the 15 moHSPC genes, which revealed two significantly enriched motifs (Figure 6D). Ingenuity Pathway Analysis (IPA) was used to identify an additional set of factors with known binding sites (right-tailed Fisher's exact test, $p < 0.05$; Figure 6E). To focus more specifically on the functional effectors of BM transplant, we next examined our data to identify genes that distinguish moHSC^{Slam} and moMPP^{Slam} from their steady-state equivalents. Pairwise analysis revealed 42 genes differentially expressed between moHSC^{Slam} and HSC^{Slam} (fold change > 1.5; FDR < 10%; Figure S6A; Table S6). This gene set was enriched for a number of functional categories, including apoptosis and cell adhesion, exocytosis and actin cytoskeleton organization, and cell motility (Figure S6C). Proximal promoter analysis of the 42 moHSC^{Slam} genes identified three enriched sequence motifs and corresponding regulators (Figure S6B). In moMPP^{Slam}, 182 genes were differentially expressed (fold change > 1.5; FDR < 10%; Figure S6D; Table S7). IPA revealed enrichment of a number of categories, including cell cycle and cancer and cell movement and immune cell trafficking, among others (Figure S6F).

Altogether, the genes identified through this analysis define a molecular signature associated with HSPC proliferation and mobilization. Importantly, HSC^{Slam} and MPP^{Slam} display remarkably similar transcription profiles during mobilization, despite inherent differences in their self-renewal potential, thereby suggesting common targets in stem and progenitor cells whose manipulation can lead to perturbed proliferation, adhesion, and migration.

Hlf Is a Positive Regulator of Multilineage Potential and Self-Renewal In Vitro

A central goal in our analysis of HSC-specific expression patterns was to identify key regulators that modulate HSC fate and function. We chose *Hlf* for functional validation because it is one of the most strikingly HSC-specific genes (Figures 4B–4D) and was predicted by module analysis to be an HSC regulator (Figure 5C). *Hlf* encodes a PAR-bZIP transcription factor that is studied principally in the context of acute leukemia involving the t(17;19) translocation that generates the oncogenic E2A-HLF fusion protein (Hunger et al., 1992; Inaba et al., 1992). Ectopic expression of *HLF* was reported to enhance the short-term xenograft potential of human lineage-negative cord blood cells, suggesting an important role in HSPC biology (Shojaei et al., 2005). We therefore constructed doxycycline-inducible *Hlf* and control lentiviruses containing an IRES-ZsGreen reporter cassette, and transduced HSCs, MPP1s, MPP2s, CMPs, GMPs and MEPs purified from mice expressing the reverse tet-transactivator, rtTA, at the Rosa26 locus (Hochedlinger et al., 2005). Transduced cells were cultured and immunostained at weekly intervals for lineage markers and CD150 (Slamf1) to monitor differentiation and evaluate the presence of primitive hematopoietic progenitors. Enforced expression of *Hlf* in HSCs caused a significant percentage of cells to maintain a lin$^-$CD150$^+$ immunophenotype during 3 weeks of ex vivo culturing, whereas control-transduced HSCs quickly lost this primitive immunophenotype and became lin$^+$CD150$^-$ (Figure 7A). Strikingly, *Hlf* was also able to induce a lin$^-$CD150$^+$ immunophenotype in a number of downstream progenitors that were initially sorted as CD150$^-$, which was maintained over several weeks of culturing (Figure 7A). After 30 days of culture in the presence of doxycycline, the *Hlf*-transduced cultures contained multiple myeloid cell types, including megakaryocytes, macrophages, granulocytes, and undifferentiated cells, whereas the control cultures contained only macrophages (Figure 7B). In an independent experiment, ectopic expression of *Hlf* or *HoxB4* in HSCs maintained mixed myeloid colony-forming potential after long-term (45 days) ex vivo culturing. In contrast,

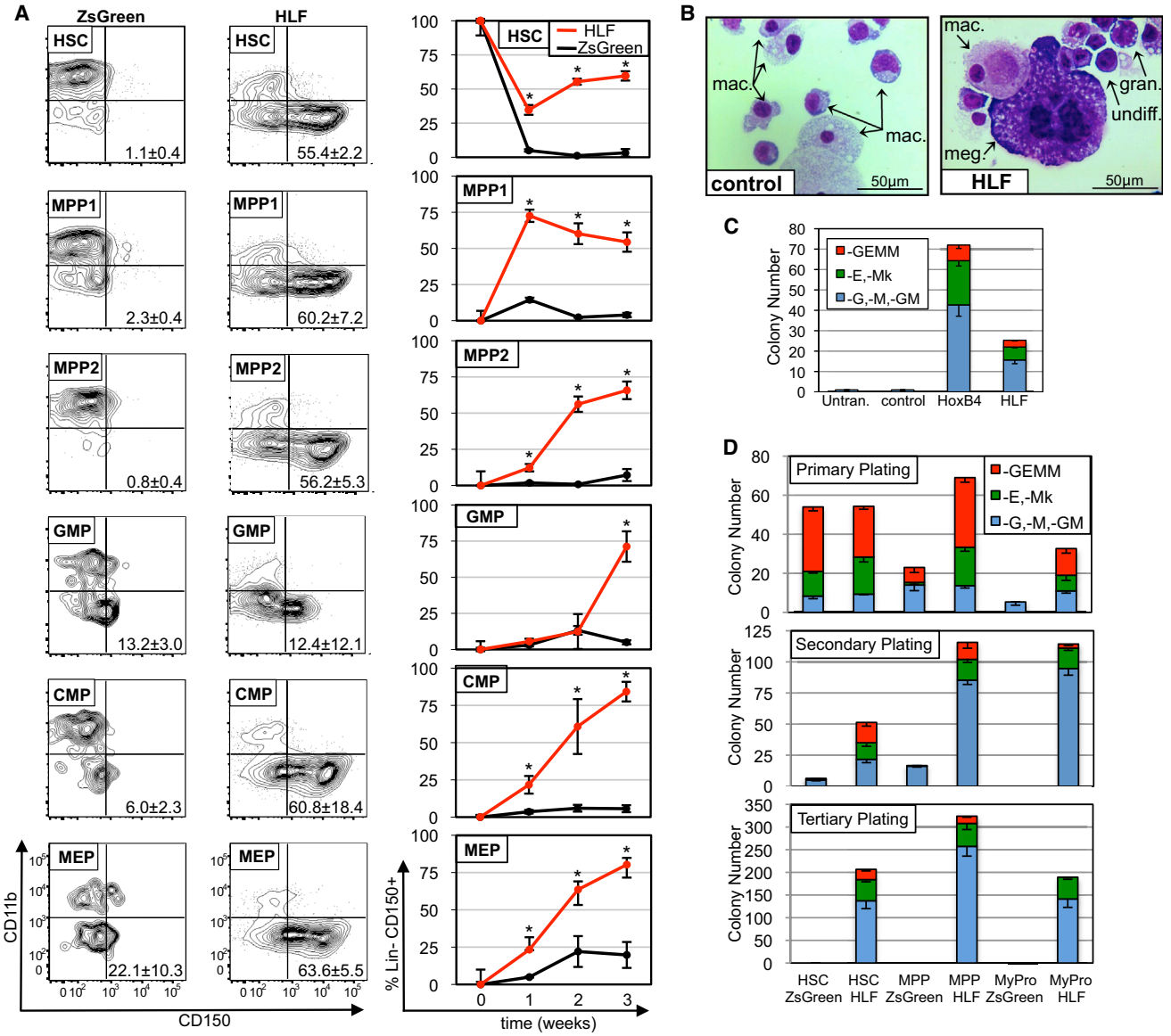


Figure 7. HLF Is a Positive Regulator of Multipotency and Self-Renewal In Vitro

(A) Representative flow-cytometry plots showing CD11b and CD150 staining (left) and the time course (right) of the indicated HSPC subsets transduced with control (ZsGreen) or HLF lentiviruses. Plots (left) were generated 2 weeks posttransduction in liquid culture. Cells were pregated on lineage markers (CD3, B220, Ter119, and Gr1). Representative experiment with three biological replicates (±SEM). *$p < 0.05$.

(B) Cytospin showing representative cell types generated by HSCs transduced with control or HLF-expressing lentiviruses and maintained in liquid culture for 30 days.

(C) Colony number and composition from HSCs transduced with control, HoxB4, or HLF-expressing lentiviruses and cultured for 45 days prior to plating. Three biological replicates per sample (±SEM).

(D) Colony number and composition upon serial plating in methylcellulose of the indicated stem and progenitor cells transduced with control or HLF-expressing lentiviruses. Three biological replicates per sample (±SEM).

See also Figure S7.

untransduced or control transduced HSCs showed limited colony-forming potential, and an inability to maintain of mixed myeloid lineage potential. Thus, ectopic expression of *Hlf* leads to the maintenance of mixed myeloid lineage potential within HSC cultures even after prolonged ex vivo culturing.

To further examine the functional potential of *Hlf*, we sorted and transduced HSCs, MPPs, and cKit$^-$ Sca1$^+$lineage$^-$ myeloid progenitors (MyPros) with *Hlf* or control virus, and assayed for colony-forming cell (CFC) activity in methylcellulose-based serial plating experiments. Both control and *Hlf*-transduced cells produced colonies in the primary plating, although *Hlf*-transduced MPPs and MyPros generated significantly more colonies (Figure 7D). Secondary and tertiary plating revealed that only *Hlf*-transduced cells continued to robustly generate colonies, whereas control-transduced cells lost activity, as expected. Importantly, quantification of colony types further revealed that *Hlf* expression conferred sustained multilineage potential, as evidenced by the presence of CFU-GEMM colonies at each plating (Figure 7D). Withdrawal of doxycycline led to loss of CFC activity, indicating that continued *Hlf* expression is necessary to sustain replating potential (Figure S7). Taken together, these experiments demonstrate that *Hlf* can impart potent, sustained self-renewal activity on HSCs and downstream progenitors during ex vivo manipulation.

DISCUSSION

HSPCs include rapidly cycling progenitor cells that produce vast numbers of effector cells on a daily basis. It was therefore not unexpected to find that genes involved in cell cycle and metabolic growth were enriched in HSPCs, but surprisingly, we also discovered that many of these genes are highly expressed in quiescent HSCs. Undoubtedly, this result is influenced in part by the fact that certain aspects of cell-cycle regulation occur posttranscriptionally. Although it is possible that the small percentage (~5%) of cycling HSCs (Passegué et al., 2005; Rossi et al., 2007; Wilson et al., 2008; Yamazaki et al., 2006) might account for all or most of the transcripts associated with cell cycle and metabolic growth, this possibility is unlikely to explain the high expression levels we observed. Robust expression of cell-cycle progression and metabolic growth genes in HSCs is consistent with the idea that, despite quiescence, these cells are primed for rapid activation, possibly as a mechanism to allow for rapid cell-cycle entry in response to acute injury or stress. Moreover, these data suggest that the balance between HSC dormancy and activation is regulated, at least in part, posttranscriptionally. In support of this, p57, the CDK inhibitor that is responsible for maintaining HSC quiescence (Matsumoto et al., 2011; Zou et al., 2011), has been shown to localize to the cytoplasm along with CyclinD2 in quiescent HSCs, and upon cytokine stimulation p57 is rapidly degraded concomitantly with translocation of CyclinD2 to the nucleus and entry into the cell cycle (Passegué et al., 2005; Rossi et al., 2007; Wilson et al., 2008; Yamazaki et al., 2006).

Delineating the transcriptional programs that underlie HSPC cell mobilization provides molecular insight into the regulation and function of cells whose robust activity is essential for the clinical success of hematopoietic cell transplantation. Retention of HSPCs within the stem cell "niche" is regulated in part by interactions between ligands expressed in the niche and receptors on the surfaces of HSPCs (such as SDF-1–CXCR4; TPO-MPL, and VLA-4–VCAM-1). G-CSF treatment is thought to attenuate these retention signals via stimulation of proteolytic enzymes to promote HSPC egress from BM into the circulation (Dar et al., 2006). Although activated myeloid cells are widely acknowledged as a primary source for such proteolytic enzymes, our analysis unexpectedly identified HSPC intrinsic upregulation of several genes encoding extracellular and transmembrane proteases, suggesting that HSPCs may produce autocrine signals that promote their migration in response to mobilizing signals. Intriguingly, many of the enriched moHSPC biological functions and molecular pathways mirror those used by immune and/or cancer cells for attachment, migration, and homing (Tables S5, S6, and S7). Further elucidation of these common pathways and the many as yet uncharacterized genes will enhance our understanding of stem and progenitor cells during mobilization, and may potentially lead to increased clinical efficacy of stem-cell-targeted therapies for hematopoietic malignancies.

Although several genes have been identified that regulate HSC self-renewal and quiescence, candidate regulators of hematopoietic multipotency remain elusive. Therefore, our identification of a large family of mostly unstudied KRAB domain-containing zinc-finger transcriptional regulators whose expression is enriched within the multipotent HSC compartment is intriguing. CH3 zinc-finger proteins bind DNA with each finger interacting with three or four bases (Urrutia, 2003). Because the genes we identified in this family encode proteins that contain three to 23 zinc fingers (average 12), they likely bind with great specificity within the genome. Strikingly, because each of these factors also contain KRAB domains, which canonically function to recruit proteins to mediate transcriptional repression, the proteins we have identified are likely to act as transcriptional repressors. Given their enriched expression in multipotent progenitors, we hypothesize that the collective activity of these factors may be involved in maintaining hematopoietic multipotency, perhaps through active repression of lineage commitment and differentiation programs. Interestingly, expression for most of these factors is not fully restricted to the multipotent stem (HSC) and progenitor (MPP) cell compartments, and instead is often maintained in one or more downstream lineages. Such

expression patterns would be consistent with the idea that individual factors function to repress commitment to defined lineages and therefore must be maintained during commitment to opposing lineages as a means of preventing aberrant activation of gene programs associated with other lineages. For example, expression of the KRAB-containing zinc-finger genes *Gm14420* and *A630089N07Rik* is maintained from HSCs and MPPs through to MEPs, but is significantly diminished in progenitors of other lineages, including GMPs, pre-B cells, and pre-T cells, suggesting that these two proteins may be involved in repressing genes associated with non-MEP cell fates. The hypothesis that these KRAB domain-containing regulators play a role in maintaining multipotency in HSPCs through suppression of differentiation pathways in either a general or lineage-specific manner remains to be experimentally tested.

The mechanisms that regulate the central properties of HSCs are not fully understood. Using the vast resource of ImmGen, we sought to identify genes with enriched expression in HSCs, reasoning that such genes might represent key regulators of stem cell fate and function. In support of this, we readily identified several known HSC regulators, including *HoxB4*, *Erg*, HoxA9, *Meis1*, *Egr1*, and *Mecom* (Orkin and Zon, 2008), as well as genes that have not previously been implicated in HSC biology. Based on its HSC-specific expression and predicted regulatory role as determined by module analysis, we identified *Hlf* as a high-priority candidate for functional validation. We found that *Hlf* endowed HSCs and downstream progenitors with enhanced self-renewal, and sustained long-term mixed myeloid lineage potential during ex vivo culturing. Interestingly, these results complement and extend a previous report examining *HLF*-expression in human HSPCs, in which ectopic expression of *HLF* led to an increase in the short-term xenograft potential of human lineage-negative cord blood cells containing HSCs and all of their downstream progenitor progeny (Shojaei et al., 2005). Our finding that HLF can impart potent and sustained self-renewal activity to HSPCs ex vivo suggests that increased self-renewal of HSPCs may underlie the observations reported by Shojaei et al. (2005).

The insights this study provides into the transcriptional regulation of HSCs, combined with the identification of HSC-specific transcription factors, could eventually lead to the development of combinatorial strategies aimed at inducing HSC potential in nonstem cells in a manner similar to that used for the reprogramming of other cell types (Graf and Enver, 2009). Moreover, our findings regarding the transcriptional programs that regulate the central properties of HSCs not only provide insights into the basic biology of these cells but may also illuminate innovative strategies to improve their clinical utility.

EXPERIMENTAL PROCEDURES

Sorting HSPCs

Immunophenotypes of HSPC subsets are shown in Table S1. Cytokine-induced mobilization of HSPCs was performed as previously described (Passegué et al., 2005). Experimental cell-sorting and processing schemes are available at https://www.immgen.org/index_content.html.

Microarray and Informatic Analysis

ImmGen V1 samples were not amplified prior to microarray hybridization, except for those cells obtained in the mobilization studies presented herein, which were amplified (Genisphere) prior to hybridization. For this reason, these data sets were normalized and analyzed independently of the broader ImmGen data set. The numbers of microarrays utilized for stem and progenitor cell populations are as follows: HSC (3), MPP1 (2), MPP2 (2), CMP (3), MEP (2), GMP (3), CLP (4), pre-B cells (3), and pre-T cells (3). In order to identify genes with enriched expression in different hematopoietic subsets, one-way ANOVA was implemented by the MATLAB function anova1. Secondary analysis was implemented by the MATLAB function multicompare. Gene lists were subjected to standard enrichment analysis through DAVID (Huang et al., 2009). For the mobilized HSC^{Slam} and MPP^{Slam} comparisons, biological functions and molecular pathway analysis association networks were generated by IPA software (v8.7; Ingenuity Systems). Significance in the data set analyzed by IPA was determined by a right-tailed Fisher's exact test ($p < 0.05$) using the whole IPA knowledge base as a reference set. To generate heatmaps, Gct files of the selected genes were visualized through GenePattern. Module analysis was done as previously described (Jojic et al., 2013).

Gene Network Prediction

A functional gene network was constructed using the full set of ImmGen microarrays (March 2010 release). The gene-by-microarray matrix of expression values was taken as input to the CLR algorithm (Faith et al., 2007). Briefly, CLR calculates a mutual information matrix of all pairwise gene-by-gene expression profiles, where an expression profile is defined as the vector of $\log_2$-transformed expression values across all ImmGen cell populations. For each individual gene, the distribution of mutual information values is Z transformed to derive a normal distribution. Background correction for each gene is applied using Stouffer's Z-score method to combine Z scores. An FDR is calculated for each of these values and an edge is drawn between two genes if the calculated FDR $< 1 \times 10^{-3}$. Cytoscape was used for network visualization (Shannon et al., 2003).

Motif Analysis

Proximal Promoter sequences (±1,000 bp of TSS) were retrieved from Ensemble BioMart (Kinsella et al., 2011) using the NCBI v37 mouse genome assembly. MEME-chromatin immunoprecipitation (MEME-ChIP) (Machanick and Bailey, 2011) was used to identify enriched sequence motifs between 6 and 30 bp.

Functional Assays

Hlf (MGI:96108) was cloned into the pHAGE2 lentivirus (Mostoslavsky et al., 2005) under a TRE promoter. Cells were double sorted for purity and transduced at 100 multiplicity of infection. For in vitro immunophenotype assays, cells were cultured in Dulbecco's modified Eagle's medium/F-12 media supplemented with doxycycline (1 μg/ml), L-glutamine, pen-strep, nonessential amino acids, beta-mercaptoethanol, 10% fetal bovine serum, and the cytokines thrombopoietin, stem cell factor, interleukin-3, and flt3L (each at 10 ng/ml). For CFC assays, cells were transduced, and cultured in liquid media for 2 days, and then transduced cells were sorted and plated in M3434 methylcellulose media (Stem Cell Technologies) at 250 cells per well. Colony number and type were quantified on day 9 or 10, followed by serial replating of 10,000 cells per well.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.07.004.

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